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A kinase-negative epidermal growth factor receptor that retains the capacity to stimulate DNA synthesis

(catalysis/phosphorylation)

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ABSTRACT **The residue proposed to serve as the catalytic base for phosphoryl transfer, Asp-813, of the human epidermal growth factor receptor (EGFR) was mutated to Ala, and the mutant receptor (D813A) was expressed in Chinese hamster ovary (CHO) cells. Partially purified D813A exhibited no detectable kinase activity in the absence or presence of EGF. A low level of EGF-stimulable phosphorylation of D813A was detectable in intact cells, apparently due to the activity of an associated Tyr kinase(s). As previously observed for kinase-inactive Lys-721 mutants, EGF binding to D813A stimulates mitogen-activated protein kinase activity. Surprisingly, and unlike results reported for Lys-721 mutants, D813A is capable of stimulating both $^{86}Rb^+$ uptake and DNA synthesis in response to EGF. These data suggest not only that Asp-813 is critical to the catalytic activity of the EGFR but also that differences may exist in the signaling properties of kinase-negative Lys-721 and kinase-negative Asp-813 EGFR mutants.**

Sequence alignment of the catalytic domains of 65 protein kinases (1) has revealed the presence of many conserved residues, which have been implicated as mediators of kinase function by scanning mutagenesis (2, 3) or chemical modification (4, 5). The importance of these residues was recently confirmed by solution of the crystal structure of the catalytic subunit of cAMP-dependent protein kinase (cAPK) (6, 7). Among these residues are the lysyl residue (Lys-72) proposed to interact with the α- and β-phosphates of MgATP (7) and the aspartyl residue (Asp-166) proposed to be the catalytic base responsible for abstraction of a proton from the serine hydroxyl group of substrates (6, 7). Chemical modification (8–10) or mutation (11–15) of Lys-721 of the epidermal growth factor receptor (EGFR), the residue homologous to Lys-72 of cAPK, abolishes kinase activity, and Lys-721 mutations (11–15) prevent activation of numerous cellular responses to EGF including DNA synthesis. Mutation of the aspartyl residue homologous to Asp-166 of cAPK in the Tyr kinases v-fps (16) and c-kit (17, 18) has also been shown to eliminate *in vitro* kinase activity. In the case of the EGFR, an antibody specific for a sequence of the EGFR including Asp-813, the residue homologous to Asp-166 of cAPK, has been shown to inhibit kinase activity (19). To test whether Asp-813 of the human EGFR is catalytically essential, we used site-directed mutagenesis to convert Asp-813 to Ala (D813A). Wild-type (WT) EGFR and D813A, expressed in Chinese hamster ovary (CHO) cells, were characterized in terms of their kinase activities and their abilities to initiate EGF-dependent signal transduction. The results indicate that Asp-813 plays an important role in the enzymatic mechanism of the EGFR protein tyrosine kinase and suggest that certain properties of the EGFR, in addition to receptor kinase activity or receptor phosphorylation, may be necessary for certain EGF-stimulated responses.

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MATERIALS AND METHODS

Materials. Murine EGF and ^{125}I-labeled EGF (^{125}I-EGF) were prepared as described (20, 21). [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 Gbq), [^{3}H]thymidine (20 Ci/mmol), and $^{86}Rb^+$ (1 Ci/g) were from DuPont/NEN. The synthetic T669 peptide (KRELVEPLTPSGEAPNQALLR) was kindly provided by R. J. Davis (University of Massachusetts Medical School). Monoclonal antibody PY-20 was from ICN. Horseradish peroxidase-conjugated monoclonal antibody RC20H was from Transduction Laboratories (Lexington, KY). EGFR antiserum was kindly provided by G. Carpenter (Vanderbilt University).

Mutagenesis and Expression. The pXER construct encoding a mutant dihydrofolate reductase (DHFR) and the human WT EGFR was kindly provided by G. N. Gill (University of California at San Diego) (11). Oligonucleotide-mediated site-directed mutagenesis was performed to change Asp-813 to Ala (23). The D813A mutation was confirmed by dideoxynucleotide sequencing using Sequenase (United States Biochemical) (23).

The pXER and pXER-D813A constructs were transfected by the Polybrene method (23) into CHO cells, CHO(DHFR$^-$), which are DHFR deficient. Stable clones were selected by using MEMα supplemented with 10% dialyzed fetal bovine serum (FBS) (Sigma; M_r 10,000 cutoff) without or with 1 μM methotrexate (MTX) (Sigma) and screened for expression of cell-surface EGFR by ^{125}I-EGF binding assays at 4°C (24). Positive clones obtained from at least two independent transfections were subjected to amplification by growth in progressively higher levels of MTX (23). Overnight treatment with 2 mM sodium butyrate (Sigma) (25) further increased receptor expression by as much as 4-fold. Clones expressing between 5.0×10^4 and 2.7×10^6 receptors per cell as estimated by ^{125}I-EGF binding assays were used in experiments as indicated.

Cell Culture. CHO(DHFR$^-$) cells (American Type Culture Collection, CRL 9096) were maintained in Ham's F12 medium containing 10% FBS. NIH 3T3 cells expressing the Dc214 EGFR truncation mutant (26), a generous gift of A. Sorkin (Vanderbilt University), were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum and gentamicin. Clones expressing either WT EGFR or D813A were maintained in MEMα containing 10% dialyzed FBS and the concentration of MTX used for

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; MAP kinase, mitogen-activated protein kinase; cAPK, cAMP-dependent protein kinase; WT, wild type; DHFR, dihydrofolate reductase; WGA, wheat germ agglutinin; IR, insulin receptor.

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selection. A431 cells were maintained in DMEM containing 5% calf serum, 20 mM Hepes (pH 7.5), and gentamicin. All cells were grown at 37°C in an atmosphere of 5% CO_2/95% air.

Cell Lysis. Confluent cell monolayers in T75 flasks were treated with 2 mM sodium butyrate overnight, washed twice with Ca^{2+},Mg^{2+}-free PBS (137.0 mM NaCl/8.0 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4), treated without or with EGF (0.50 μg/ml), and solubilized in 1 ml of TGH buffer [150 mM Hepes, pH 7.5/1% Triton X-100/10% (vol/vol) glycerol/1 mM EGTA/0.1 mM phenylmethylsulfonyl fluoride (PMSF)/aprotinin (10 μg/ml)/leupeptin (10 μg/ml)] without or with 1 mM Na_3VO_4 at 4°C for 20 min with rocking. Lysates were cleared by centrifugation at 16,000 × *g* for 10 min at 4°C and stored at −70°C until needed.

Wheat Germ Agglutinin (WGA)-Agarose Chromatography. WGA-agarose (Vector Laboratories) washed with WGA buffer (20 mM Hepes, pH 7.5/200 mM NaCl/1% Triton X-100/10% glycerol) was added in a 1:10 (vol/vol) ratio to cell lysates supplemented with 10 mM $MgCl_2$. After rocking at 4°C overnight, the resin was washed three times in WGA buffer containing 10 mM $MgCl_2$. Bound glycoproteins, including the EGFR, were eluted from the resin by two 30-min incubations with rocking at 4°C in TGH buffer containing 3 mM *N,N',N''*-triacetylchitotriose (Sigma).

Immunoprecipitations. Protein A-Sepharose CL-4B (Pharmacia) was incubated in HTNG buffer (50 mM Hepes, pH 7.5/0.1% Triton X-100/50 mM NaCl/10% glycerol) containing EGFR antiserum (1:10 dilution) overnight at 4°C. The resin was washed four times with cold HTNG buffer, added to cell lysate [1:20 (vol/vol) ratio], and incubated with rocking for 1–2 h at 4°C. Immunoprecipitates were then washed extensively in HTNG buffer containing either 50 mM (low salt) or 500 mM (high salt) NaCl as indicated in the figure legends.

***In Vitro* Phosphorylation Assays.** Samples were treated without or with EGF for 10 min at room temperature, followed by 10 min on ice. Assays were initiated by the addition of phosphorylation buffer without or with [γ-^{32}P]ATP to give final buffer concentrations of 150 mM Hepes, pH 7.5/5 mM $MgCl_2$/1 mM $MnCl_2$/10 μM Na_3VO_4/20 μM ATP. Reaction mixtures containing WGA eluates were terminated after 2 min on ice by the addition of 4× sample buffer (0.25 M Tris·HCl, pH 6.8/8% SDS/40% glycerol/0.20 M dithiothreitol/0.004% bromophenol blue) and heated for 5 min at 95°C. Reaction mixtures containing immunoprecipitates were terminated after 15 min on ice by the addition of 1 ml of HTNG buffer containing 10 mM EDTA, pelleted, resuspended in 1× sample buffer, and heated for 5 min at 95°C. Samples were then separated by SDS/PAGE followed by either autoradiography or immunoblot analysis.

Electrophoresis and Immunoblotting. SDS/PAGE was performed as described by Laemmli (27). Semidry transfer of proteins to nitrocellulose (Micron Separations, Westboro, MA) was accomplished with a Hoefer semiphor transfer unit and the accompanying protocols. Autoradiographs of dried gels or immunoblots developed by enhanced chemiluminescence (ECL) using ECL (Amersham) or Renaissance (DuPont/NEN) were performed with Kodak X-Omat AR film.

Mitogen-Activated Protein (MAP) Kinase Assay. The activation of MAP kinase by CHO cells expressing WT EGFR or D813A was measured as described by Selva *et al.* (15). Briefly, confluent cell monolayers were treated without or with EGF (100 ng/ml) for 5 min at 37°C and lysed in 25 mM Hepes, pH 7.5/5 mM EDTA/50 mM NaF/100 μM Na_3VO_4/1 mM PMSF/aprotinin (10 μg/ml)/leupeptin (10 μg/ml). The lysates were cleared by centrifugation at 170,000 × *g* for 20 min at 4°C. Phosphorylation reactions were performed in triplicate using 15-μl aliquots of each lysate in the absence (control) or presence of 25 μg of T669 peptide as described (15). Protein assays (Bio-Rad) were performed to allow normalization of lysates.

$^{86}Rb^+$ Uptake Assay. The accumulation of $^{86}Rb^+$ by CHO cells expressing WT EGFR or D813A was measured as described by Selva *et al.* (15). Cell monolayers (16-mm wells; ≈50% confluent) were rinsed twice with KRH buffer (120 mM NaCl/6 mM KCl/1.2 mM $MgSO_4$/1 mM $CaCl_2$/25 mM Hepes, pH 7.4) supplemented with 30 μM bovine serum albumin (BSA) and then incubated in 0.5 ml of KRH/BSA for 30 min at 37°C. After treatment of triplicate wells without or with EGF (100 ng/ml) for 5 min, $^{86}Rb^+$ (1 μCi) was added to each well. After 5, 10, or 15 min, each set of triplicate wells was rinsed three times with cold 1× Hanks' balanced salt solution (Sigma) and solubilized in 0.5 ml of 0.1% SDS for 1 h at 37°C. Ecolite (10 ml) (ICN) was added to each sample, and the radioactivity was measured in a Beckman LS 7500 liquid scintillation counter.

DNA Synthesis. Cells were seeded at a density of 1.5 × 10^4 cells per 16-mm well in the appropriate medium for each cell type. After 48 h, the cells were shifted into medium containing 0.1% FBS for 48 h. Cells were then treated with EGF at 0, 10, or 100 ng/ml for 20 h. The conditioned medium of each well was supplemented with 1 μCi of [^{3}H]thymidine (final concentration, 2.5 μM) and incubated at 37°C for 4 h. The plates were then put on ice, washed three times with ice-cold PBS (137 mM NaCl/8.8 mM Na_2HPO_4/2.7 mM KCl/1.5 mM KH_2PO_4/0.9 mM $CaCl_2$/0.5 mM $MgCl_2$), and incubated with 0.5 ml of ice-cold 10% trichloroacetic acid (TCA) for 1 h on ice. After washing two times with ice-cold 10% TCA, the acid-insoluble fraction was solubilized in 0.5 ml of 0.2 M NaOH/0.1% SDS for 1 h at 37°C. The samples were neutralized with 0.5 ml of 2 M Tris·HCl (pH 7.5) and mixed with 10 ml of Ecolite. Incorporation of [^{3}H]thymidine was measured in a Beckman LS 7500 liquid scintillation counter.

RESULTS AND DISCUSSION

We mutated Asp-813 of the EGFR to Ala and expressed both the mutant (D813A) and WT EGFR in CHO(DHFR$^-$) cells, which lack detectable endogenous EGFR (11, 28–30). ^{125}I-EGF binding assays performed on isolated clones demonstrated that WT EGFR or D813A was expressed on the cell surface at levels up to 2.7 × 10^6 receptors per cell. To determine the effect of the mutation on *in vitro* kinase activity, we examined the ability of D813A either to autophosphorylate or to be transphosphorylated by the kinase-active EGFR mutant, Dc214, which is truncated after Asn-972 and thus lacks all five known autophosphorylation sites (26). A431 cells, untransfected CHO(DHFR$^-$) cells, CHO cells expressing either WT EGFR or D813A (M_r ≈ 170,000), and NIH 3T3 cells expressing the Dc214 EGFR truncation mutant (M_r ≈ 140,000) were solubilized and subjected to WGA-agarose chromatography. WGA eluates from untransfected CHO(DHFR$^-$) cells displayed no detectable EGF-stimulable autophosphorylation activity (Fig. 1 *A* and *B*). WT EGFR from A431 cells or from transfected CHO cells were capable of EGF-stimulable autophosphorylation as detected by ^{32}P labeling (Fig. 1*A*) or PY-20 immunostaining (Fig. 1*B*) of the 170- and 150-kDa bands corresponding to intact and proteolyzed EGFR (31), respectively (Fig. 1*C*). No receptor phosphorylation, however, was detected in kinase reactions of D813A eluates (Fig. 1 *A* and *B*), even when reactions were performed with physiological (2 mM) ATP concentrations (data not shown). This apparent lack of activity of D813A is even more striking given the apparent ratio of D813A to WT EGFR (Fig. 1*C*). These data suggest that partially purified D813A is unable to autophosphorylate *in vitro*.

Since Dc214 lacks the five known autophosphorylation sites, no ^{32}P labeling (Fig. 1*A*) or PY-20 immunostaining (Fig.

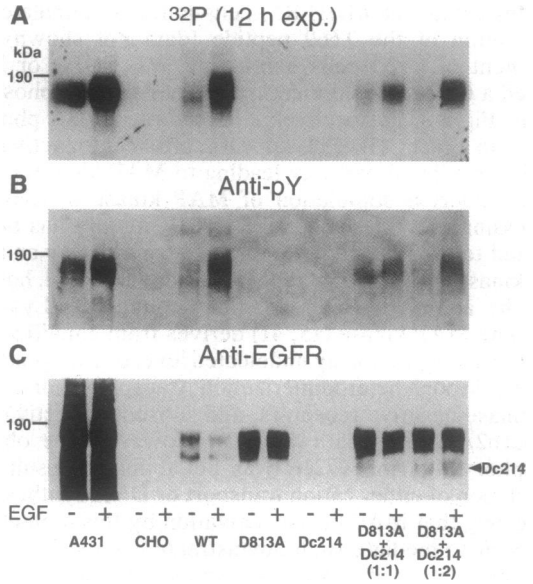

FIG. 1. *In vitro* kinase activity of D813A. WGA eluates prepared from A431 cells, untransfected CHO(DHFR$^-$) cells, CHO cells expressing either WT EGFR (2.4×10^6 receptors per cell) or D813A (2.7×10^6 receptors per cell), and NIH 3T3 cells expressing the Dc214 mutant (5.0×10^5 receptors per cell) were incubated either individually (A431, CHO, WT, Dc214, or D813A) or in combination [D813A plus Dc214 in a 1:1 or 1:2 (vol/vol) ratio] without or with 1 μM EGF for 10 min at room temperature, followed by 10 min on ice. Radioactive or nonradioactive kinase assays were then performed on duplicate samples as described, separated by SDS/PAGE, and subjected to autoradiography (*A*) or PY-20 immunoblot analysis (*B*), respectively. (*C*) The PY-20 immunoblot shown in *B* was stripped according to Amersham's ECL protocol and reprobed with EGFR antiserum. Similar results were obtained in two independent experiments.

1*B*) of the corresponding band (Fig. 1*C*) was observed. EGF does stimulate the *in vitro* Tyr phosphorylation of the band corresponding to D813A in the presence of an equal or 2-fold excess volume of Dc214 WGA-agarose eluate (Fig. 1 *A* and *B*). These results imply that D813A can form heterodimers with Dc214 and act as a substrate for transphosphorylation by Dc214. Cross-linking studies on cell extracts, performed as described (32), confirmed that both WT EGFR and D813A undergo EGF-stimulable homodimerization (data not shown). That D813A is expressed on the cell surface, can bind EGF, can be transphosphorylated, and can dimerize strongly implies that mutation of Asp-813 does not perturb the overall structure of the molecule.

We also examined the *in vitro* kinase activity of D813A toward two exogenous peptide substrates: tyrsub, a high-affinity substrate for the EGFR (33), and AT II, a well-characterized substrate for Tyr kinases (34). In contrast to WT EGFR, D813A is incapable of detectably phosphorylating either tyrsub or AT II (data not shown). The failure of D813A to autophosphorylate or to phosphorylate exogenous peptide substrates *in vitro* suggests that Asp-813 is critical for EGFR protein tyrosine kinase activity, consistent with the hypothesis that Asp-813 functions as the catalytic base in phosphoryl transfer.

In contrast to *in vitro* phosphorylation assays in which no phosphorylation of D813A was observed, D813A is phosphorylated in intact cells on tyrosyl residues in an EGF-stimulable manner (Fig. 2*A*), albeit to only a small fraction of that observed for WT EGFR. This phosphorylation of D813A in intact cells may be due either to a low level of intrinsic D813A kinase activity not detectable with *in vitro* assays or to an endogenous tyrosyl kinase(s). Candidates for such an endogenous tyrosyl kinase(s) include (*i*) an EGF-stimulable

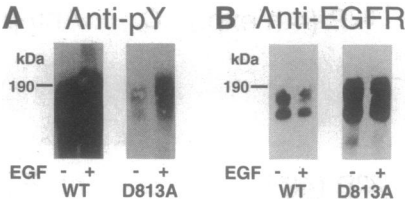

FIG. 2. Phosphorylation of D813A in intact cells. CHO cells expressing either WT (2.4×10^6 receptors per cell) or D813A (2.7×10^6 receptors per cell) were treated in the absence or presence of EGF (0.50 μg/ml) in DMEM for 5 min at 37°C, solubilized in TGH buffer supplemented with 1 mM Na_3VO_4, and immunoprecipitated with EGFR antiserum. Immunoprecipitates were separated by SDS/PAGE and subjected to immunoblot analysis using horseradish peroxidase-conjugated RC20H (*A*). The anti-pY immunoblot was then stripped and reprobed with EGFR antiserum (*B*). Similar results were obtained in three independent experiments.

cellular tyrosyl kinase, (*ii*) EGFR-related tyrosyl kinases such as ErbB2 or ErbB3, and (*iii*) endogenous hamster EGFRs. Although ^{125}I-EGF binding assays (data not shown; refs. 11 and 28–30) and Northern blot analysis (28) fail to detect endogenous EGFR in CHO cells, a recent report suggests that these techniques may lack the sensitivity required to measure extremely low levels of receptor (35). Likewise, although immunoblot analysis of CHO cells reportedly failed to detect ErbB2 and ErbB3 (15), undetectable levels of EGFR-related receptors might be responsible for the observed EGF-stimulable tyrosyl phosphorylation of D813A in intact cells. Alternatively, Selva *et al.* (15) recently reported EGF-stimulable tyrosyl phosphorylation of the kinase-negative K721R EGFR mutant in intact CHO cells, which they attributed to phosphorylation of the receptor by an associated kinase. In addition, an EGF-stimulable cellular tyrosyl kinase that is separable from the EGF receptor has also been described in mouse B82L cells (36).

To determine whether EGF-stimulable Tyr phosphorylation of D813A in intact cells was due to the activity of an associated protein tyrosine kinase(s), cells expressing either WT EGFR or D813A were pretreated with or without EGF, solubilized under low- or high-salt conditions, immunoprecipitated, and subjected to autophosphorylation assays in the presence of EGF. Extensive phosphorylation of WT EGFR was observed after either low- or high-salt lysis and immunoprecipitation (Fig. 3 *A* and *B*). However, under low-salt conditions, only a very low level of EGF-stimulable phosphorylation of D813A was observed. Moreover, high-salt lysis and immunoprecipitation effectively eliminated phosphorylation of D813A, suggesting that in intact CHO cells an EGFR-associated protein kinase(s) is responsible for the overwhelming majority, if not all, of the observed EGF-stimulable phosphorylation of D813A. Anti-phosphotyrosine immunoblot analysis (Fig. 3*C*) indicates that phosphorylation of D813A by the associated kinase(s) occurs primarily on tyrosyl residue(s). The difference in the level of phosphorylation of D813A in intact cells stimulated with EGF at 37°C for 5 min (Fig. 2) as compared to 4°C for 1 h (Fig. 3 *C* and *D*) may reflect a temperature dependence of the interaction of the associated kinase(s) with the EGFR. It should be noted that no other EGF-stimulable Tyr phosphorylated bands were observed in the lanes corresponding to immunoprecipitates of D813A in Fig. 3, suggesting that if the associated Tyr kinase is capable of autophosphorylation, then it is present at a level not detectable by these methods. Alternatively, activation of this kinase may not require and/or result in its autophosphorylation but rather may stem from the interaction of the kinase with the stimulated mutant EGFR. Although we cannot absolutely exclude the possibility that endogenous EGFR or EGFR-related kinase(s) may be re-

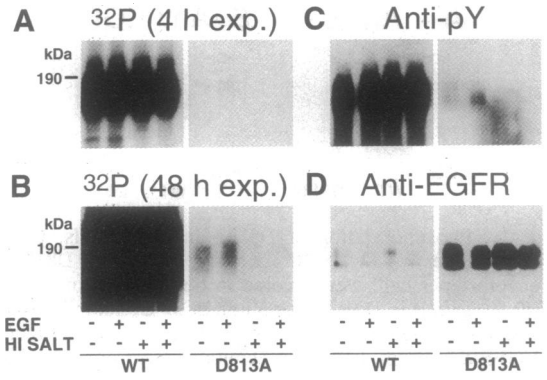

FIG. 3. Phosphorylation of D813A by an associated kinase. CHO cells expressing either WT EGFR (2.4×10^6 receptors per cell) or D813A (2.7×10^6 EGFR per cell) were treated in the absence or presence of EGF (0.50 μg/ml) for 1 h at 4°C. The cells were solubilized in a modified TGH buffer containing 25 mM Hepes, 1 mM Na_3VO_4, and either 50 mM NaCl (low salt) or 500 mM NaCl (high salt) and immunoprecipitated as described. After extensive washing with low- or high-salt HTNG buffer, each immunoprecipitate was split into two aliquots. To ensure identical assay conditions for all samples, each aliquot was washed with low-salt HTNG prior to phosphorylation assays. Samples were then incubated with low-salt HTNG containing 1 μM EGF for 15 min at room temperature followed by 10 min on ice. Radioactive (10 μCi per assay) or nonradioactive kinase assays were performed on the duplicate sets of samples for 15 min on ice, and the samples were subjected to SDS/PAGE followed either by autoradiography (*A* and *B*) or by immunoblot analysis with PY-20 (*C*). (*D*) The PY-20 immunoblot was stripped and reprobed with EGFR antiserum. Similar results were obtained in five independent experiments.

sponsible for the observed phosphorylation of D813A, this possibility is rendered less likely by the observation that this kinase activity is removed by high-salt lysis and washing. Furthermore, the associated kinase activity was also removed by high-salt washing of membranes prepared from cells expressing D813A, suggesting that the kinase responsible for the observed D813A phosphorylation is not a transmembrane protein (data not shown). The identity of the associated kinase(s) and its relationship to the associated kinase(s) observed in CHO cells (15) and in B82L cells (36) remain to be determined.

The ability of the D813A mutant to stimulate MAP kinase activity in response to EGF was determined by measuring the EGF-stimulable phosphorylation of T669, a synthetic peptide based on the sequence surrounding Thr-669 of the EGFR, a residue that is known to be phosphorylated by a member(s) of the MAP kinase family (37–40). While EGF treatment of untransfected CHO(DHFR$^-$) cells failed to stimulate phosphorylation of the T669 peptide (data not shown), EGF treatment of CHO cells expressing WT EGFR or D813A caused a 60- or 20-fold increase in T669 peptide phosphorylation (Fig. 4*A*), respectively. Therefore, D813A phosphorylation in intact CHO cells appears sufficient for activation of the Ras-mediated pathway leading to MAP kinase.

The reported stimulation of MAP kinase in CHO cells expressing the kinase-negative K721R mutant has been attributed to the phosphorylation of this mutant by an associated kinase (15). A recent report (35) has suggested, however, that the ability of EGF receptors mutated at Lys-721 to stimulate MAP kinase (15, 41) derives from amplification of signals from a previously undetected level of endogenous WT EGFRs through heterodimerization, transphosphorylation of the kinase-negative receptors, and subsequent activation of the Grb2/Sos/Ras pathway (42). However, the observed phosphorylation of K721R was not found to result in the stimulation of either cation transport or DNA synthesis (15), suggesting that MAP kinase activation by this mutant is not sufficient to mediate such downstream events. In contrast, EGF stimulation of CHO cells expressing either WT EGFR or the kinase-negative D813A mutant produces a 60% increase in the uptake of the K^+ tracer $^{86}Rb^+$ (Fig. 4*B*), while having no effect on untransfected cells (data not shown).

Furthermore, though EGF had no effect on [^{3}H]thymidine incorporation by untransfected CHO(DHFR$^-$) cells (data not shown), it stimulated [^{3}H]thymidine incorporation 3-fold in cells expressing WT EGFR at 3.0×10^5 receptors per cell (Fig. 4*C*). In CHO cells overexpressing WT EGFR (2.4×10^6 receptors per cell), however, EGF seemed to inhibit DNA synthesis (data not shown), an observation consistent with the correlation between overexpression of EGFR and EGF-mediated inhibition of growth reported for A431 cells (43) and numerous squamous cell carcinomas in culture (44). Unexpectedly, in cells expressing the kinase-negative D813A mutant at 2.7×10^6 receptors per cell, EGF stimulated [^{3}H]thymidine incorporation 3- to 6-fold (Fig. 4*C*). Similar results were obtained with D813A clones derived from several independent transfections, including a clone expressing as few as 5.0×10^4 receptors per cell (Fig. 4*C*). Our observation that EGF can stimulate DNA synthesis in a clone expressing 5×10^4 D813A receptors per cell nearly as effectively as in a clone expressing 2.7×10^6 receptors per cell argues against the stimulation arising from a high dose of low-activity receptors. The contrast between our results with the D813A mutant and those reported for the K721R mutant (15) suggests that the phosphorylation of the receptor, while sufficient to stimulate MAP kinase, is insufficient to stimulate DNA synthesis.

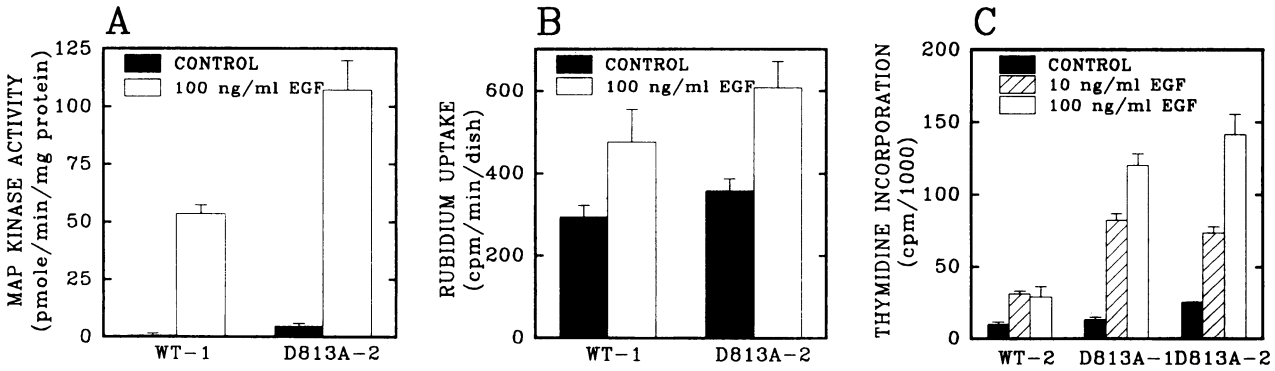

FIG. 4. Signaling properties of the kinase-negative D813A mutant. Comparison of the effects of EGF treatment of CHO cells expressing WT EGFR (WT-1, 2.4×10^6 receptors per cell; WT-2, 3.0×10^5 receptors per cell) or D813A (D813A-1, 5×10^4 receptors per cell; D813A-2, 2.7×10^6 receptors per cell). The abilities of both WT EGFR and D813A to stimulate MAP kinase activity (*A*), $^{86}Rb^+$ uptake (*B*), and DNA synthesis (*C*) in response to EGF were assessed as described. Data presented are means ± SD of triplicate determinations obtained in one experiment. Similar results were obtained in at least three separate experiments.

The stimulation of ionic fluxes is reportedly required for the stimulation of DNA synthesis (45, 46), suggesting that the reported inability of K721R to stimulate DNA synthesis (15) compared to our observations with D813A might be associated with their differing abilities to activate cation transport. Therefore, D813A may possess a signaling property or function lacking in the K721R mutant that is necessary for the EGF-mediated pathway(s) leading to DNA synthesis.

One property that may distinguish between these different kinase-negative mutants may be their abilities to bind ATP. It should be emphasized that K721A (35), K721M (35, 41), and K721R (15) receptors are mutated at the lysyl residue of the ATP-binding site (10) proposed to interact with the α- and β-phosphates of ATP (7, 47). However, the D813A receptor is mutated at the aspartyl residue proposed to serve as the catalytic base and, therefore, may retain the capacity to bind ATP. A role for ATP, aside from its role in phosphoryl transfer, is not unprecedented for protein kinases. It has been demonstrated that ATP allosterically stabilizes the high-affinity interaction of the R^1 subunit and/or the protein kinase inhibitor peptide with the catalytic subunit of cAPK (48). It is conceivable that ATP binding to the kinase-inactive D813A mutant may serve to stabilize interaction(s) with downstream effectors necessary for EGF stimulation of pathways acting in concert with or parallel to the SH2-mediated signal transduction cascade.

Recent observations on the insulin receptor (IR) kinase provide further support for this hypothesis. IR mutated in a region of the kinase domain thought to be involved in substrate binding, rather than ATP binding, has been expressed in Rat-1 cells, which contain a low level of endogenous WT IR (22). This IR deletion mutant was unable to autophosphorylate *in vitro*, and phosphorylation of the IR substrate IRS-1 in mutant-transfected cells was similar to the results obtained with the parental Rat-1 cells (22). However, insulin stimulation of Rat-1 cells expressing the mutant IR did result in enhanced DNA synthesis (22).

It is undisputed that the kinase activity and/or phosphorylation of the EGFR are important in mediating receptor interactions with SH2 proteins leading to Ras and MAP kinase activation. However, the results presented here for the kinase-negative D813A EGFR mutant, as well as the observations for the IR deletion mutant, suggest that receptor Tyr kinases may activate signaling pathways necessary for full growth factor-induced responses not exclusively via intrinsic kinase activity *per se* but rather via properties such as ATP binding that may be intimately related to this function.

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